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DIAGNOSTIC AND THERAPEUTIC METHODS IN AUTOIMMUNE DISEASE

FIELD OF THE INVENTION

The invention is in the field of pharamacogenomics, particularly the utilization of genetic alleles as diagnostic markers.

BACKGROUND OF THE INVENTION

Rheumatoid arthritis is a chronic polyarticular inflammatory disease with a variable course and outcome (Combe et al., 1995, *Br. J. Rheumatol.* 34: 529). Clinical expression ranges from a mild, non-deforming arthropathy with little long-term disability to severe, incapacitating, erosive articular destruction which may be refractory to conventional disease modifying agents (Schiff, 1997, *Am. J. Med.* 102 (suppl 1A): 11S-15S). Prediction of disease progression is imprecise, and is often based on a combination of demographic, clinical and laboratory factors, including low socioeconomic status and educational levels, severe initial disease activity, systemic manifestations and extra-articular features, the early appearance of joint erosions, an elevated erythrocyte sedimentation rate, C-reactive protein and the presence of rheumatoid factor. There is epidemiological evidence that there is a genetic relationship between loci in the major histocompatibility complex (MHC) class II region and disease susceptibility and severity (Reveille, 1998, *Curr. Op. Rheumatol.* 10: 187; Nepom et al., 1996, *J.Rheumatol.* 23 (suppl 44): 5; Weyand and Goronzy, 1995, *Curr. Op. Rheumatol.* 7: 206).

Interferon gamma is a homodimeric 34 Kd peptide. IFN gamma may be secreted by T-lymphocytes under certain conditions of activation and by NK cells (Boehm, U., et al. 1997). IFN gamma binds to cell surface receptors on cellular targets including mononuclear phagocytes, endothelial cells and NK cells, and is thought to play an important role in the coordinated regulation and expression of the immune response through the stimulation or repression of genes (Farrar, M.A. et al., 1993; Revel, M. et al., 1986). IFN gamma appears to be produced by T-cells infiltrating the inflamed synovium and may be secreted into the joint space, but the role of this peptide in the progression of the articular injury in arthritis remains controversial (Feldemann, M. et al. 1996).

IFN gamma is encoded by a gene which in humans is mapped to 12q24 on chromosome 12. The known sequence of the gene consists of 4 exons with 3 intervening regions. A variable length dinucleotide repeat polymorphism has been described in humans and lower primates within the first intron of this gene, between positions 1349 and 1373. The number of alleles reported at this microsatellite region appears to vary according to the detection methodology employed to characterize it (Ruiz-Linares A, 1993; Awata, T. et al. 1994; Pravica, V. et al., 1998).

The human leukocyte antigens (HLA) are a family of polymorphic cell-surface proteins that are involved in intercellular interactions in the immune system. The HLA genes are part of the major histocompatibiiity complex (MHC). The HLA proteins are designated HLA-A, -B, -DR, -DQ and -DP. The HLA-A, -B and -C proteins are described as Class I HLA proteins, while the HLA-DR, -DQ and -DP proteins are described as Class II proteins, and are composed of two polypeptide chains, an alpha chain and a highly polymorphic beta chain.

The Class II HLA proteins are expressed on the cell surface of macrophages, B-cells and activated T-cells, where they are thought to be involved in binding and presenting antigens to helper T-lymphocytes (see Giles and Capra, 1985, Adv. Immunol. 37:1). The Class II DP, DQ and DR genes are located in separate regions of the MHC (Trowsdale *et al.*, 1985, Imunol. Rev. 85:5). In the DR region, the DRA locus encodes the alpha chain and five different DRB loci encode the beta chain: DRB1, DRB2 (now known as DRB6), DRB3, DRB4, and DRB5.

The Class II protein genes have been segregated into a number of known haplotypes (the specific allele combination at multiple loci on the same chromosome, see for example Dupont, 1989, Hum. Immunol. 26:3), such as the DR4 haplotype which may be associated with rheumatoid arthritis. Some efforts have been made to determine which locus within the DR4 haplotype is most tightly associated with predisposition to rheumatoid arthritis (Zanelli *et al.*, 1998, Immunogenetics 48:394-401). A complex interrelationship of loci appears to be involved in various aspect of rheumatoid arthritis, including a 'shared epitope' Q(K/R)RAA at amino acids 70-74 of the *DRB1* encoded peptide. This shared epitope has been associated

with severe rheumatoid arthritis (Gregerson *et al.*, 1987, Arthritis Rheum 30:1205-1213; Williams *et al.*, 1993, DNA and Cell Biology 12(5):425-434), although its frequency in the normal population has been suggested to preclude its use as a positive indicator of disease prognosis (Khani-Hanjani *et al.*, 1998, Abstract 293, Poster Session B, American College of Rheumatorolgy 62nd National Meeting). The role of specific residues within the shared epitope of *DRB1* has been investigated (Zanelli *et al.*, 1997, J. Immunol 158:3545-3551; Wucherpfennig *et al.*, 1995, Proc. Natl. Acad. Sci. 92:11935-11939).

SUMMARY OF THE INVENTION

In one aspect, the invention provides a method of diagnosis. The diagnostic method may include steps of identifying a patient at risk of an arthritis, the patient having an interferon gamma gene. The patient may be tested to characterize a polymorphism in a first intron of the interferon gamma gene. The polymorphism may comprise a variable length dinucleotide repeat region within the first intron, and the dinucleotide repeat region may be located at least partly between nucleotides 1349 and 1373 in the interferon gamma gene. The method may be carried out so as to be capable of identifying alleles such as the 126 bp allele and the 122 bp allele, as further described herein. The polymorphisms may be distinguished based on a difference in the number of CA repeats in a portion of the first intron of the interferon gamma gene. To characterize the polymorphism, a region of the first intron may be amplified, such as a region comprising a variable length dinucleotide repeat.

The use of an allele of an interferon, gamma gene as described herein may provide prognostic information with respect to the likelihood of particular clinical outcomes for the patient, and as a result may be utilized to modify treatment regimens. In particular, the presence of alleles associated with relatively severe disease, such as the 126 bp allele, may be taken as an indication that aggressive therapy should be pursued relatively early in the progression of the disease.

In one aspect, the invention involves the identification of high and low risk interferon gamma alleles. In another aspect, the invention involves a further refinement of patient differentiation involving the use of an HLA locus in conjunction with the IFN locus.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a risk analysis tree showing the association between the severity of RA (severe or mild disease) and the IFN gamma (shown by the arrow labelled "IFN") and *HLA-DRB1* (shown by the arrow labelled "HLA") genotype of the patient.

Figure 2 shows the HLA-DRB1 amino acid sequences 70-74 for both alleles for patients with severe RA.

Figure 3 shows the HLA-DRB1 amino acid sequences 70-74 for both alleles for patients with mild RA.

DETAILED DESCRIPTION OF THE INVENTION

In one aspect, the invention discloses a correlation between two alleles, designated 126 bp and 122 bp, and the occurrence of particular disease states in arthritis. There is a positive correlation between the occurrence of the 126 bp allele and severe rheumatoid arthritis. There is a negative correlation between the occurrence of the 122 bp allele and severe rheumatoid arthritis. For each allele, there is a corresponding and reverse correlation with relatively mild rheumatoid arthritis. One aspect of the present invention therefore provides pharmacogenomic methods to assist in diagnosis of arthritis disease, including prediction of disease severity and selection of therapy regimens in particular patients.

In another aspect, the invention discloses embodiments of partial DNA sequences corresponding to the 122 bp, 124 bp and 126 bp alleles. Alleles which share a particular PCR fragment length, such as the 122 bp alleles, need not be identical in all other respects. In effect, there may be a 'family' of alleles characterized by a particular PCR amplification fragment size. Sequencing of individual embodiments of the alleles of interest produced the following sequence information (in which "N" indicates that it was not possible to unambiguously identify the base at the relevant position):

A 122 bp fragment partial sequence:
AACCACAAATNCAATNCTCACACACACACACACACACACACACCC
NNANATTTTGGAAACNANCTTTAAANCCNCNNAAAAAAANNCCCN
ANAGNANGGT

A 124 bp fragment partial sequence:
AAAACCACAAAATTCAAATNCNCACACACACACACACACACAC
ACNCCCANATTTTTGNNAANANNCTTTTAAACCCCTNAAAAAAAAN
CCCCANANGNGAGNGGGGAT

A 126 bp fragment partial sequence:
AAANCCACAAATTCAAATNCACACACACACACACACACACACACA
CACCCACANATTTTTGGAAACNANCTTTAAANCCCCNNAAAAAAA
ACCCCCAANAGGGGANGGGGATN

The various alleles have been found to have different numbers of CA repeats according to their fragment size, as follows:

120 bp allele: 11 CA repeats
122 bp allele: 12 CA repeats
124 bp allele: 13 CA repeats
126 bp allele: 14 CA repeats
128 bp allele: 15 CA repeats
130 bp allele: 16 CA repeats

Hutchinson et al., 1999, *Transplant Proc.*, 31(1-2): 734, indicate that the allele containing the 122 bp fragment, is associated with high levels of IFN-gamma production, while other alleles, including the 126 bp allele, are associated with low levels of IFN-gamma production. Accordingly, in one aspect, the invention provides for a genotyping assay to identify IFN-gamma alleles that are associated with low levels of IFN-gamma production, to provide an indication that the patient having the allele is likely to suffer from severe rheumatoid arthritis. Conversely, the invention provides assays for identifying IFN-gamma alleles associated with high levels of IFN-gamma production to provide an indication that the patient having such an allele is less likely to suffer from severe rheumatoid arthritis.

The invention may be utilized in patients identified as at risk of an arthritis, such as patients diagnosed by a medical practitioner as suffering from RA. Patients may for example be identified as at risk of an arthritis on the basis epidemiological criteria such as sex, age, socioeconomic factors or family history, on the basis of which an assessment may be made that the patient is more likely than other persons to suffer from an arthritis. Physicians typically diagnose RA based on the overall pattern of symptoms, medical history, physical

exam, X-rays and tests for rheumatoid factor or established genetic markers such as HLA-DR4. Typical symptoms of patients at risk of RA may include: general fatigue, soreness, stiffness and aching, with pain and swelling typically occurring in the same joints on both sides of the body and starting in the hands or feet, particularly the wrist and many of the hand joints. Other diagnostic symptoms may include rheumatoid nodules. The diagnosis of the patient as being at risk of an arthritis may also comprise identifying symptoms such as the following: joint erosions, elevated erythrocyte sedimentation rate, C-reactive protein, polyarticular disease, joint deformities, radiological evidence of subchondral erosions, extra-articular arthritis or the presence of rheumatoid factor. Patients may be identified as at risk by virtue of an inadequate response to one or more arthritis therapies, such as an inadequate response to DMARDs (disease-modifying antirheumatic drugs) or other medicaments for treating the arthritis.

In one aspect, the invention provides a method of treating a patient having an interferon gamma gene, comprising testing the patient to characterize a polymorphism in the interferon gamma gene; and, treating the patient for an arthritis if the polymorphism indicates that the patient is at risk of an arthritis. The polymorphism in the interferon gamma gene may be in the length of the dinucleotide repeat region within the first intron. The presence of the high risk allele of the present invention may for example be taken as indicative of susceptibility to arthritis or to a more severe form of arthritis.

In accordance with various aspects of the invention, a patient may be treated for an arthritis. For example, treating a patient for RA may comprise administering to the patient an effective amount of a medicament. An effective amount of a medicament may be a therapeutically effective amount or a prophylactically effective amount. A "therapeutically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic result, such as reducing signs and symptoms of RA and delaying structural damage of RA . A therapeutically effective amount of a therapeutic may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the therapeutic to elicit a desired response in the individual. Dosage regimens may be adjusted to provide the optimum therapeutic response. A therapeutically effective amount is also typically one in which any toxic or detrimental effects of the therapeutic are

outweighed by the therapeutically beneficial effects. A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result, such as reducing signs and symptoms of RA and delaying structural damage of RA . A prophylactic dose may be used in subjects prior to or at an earlier stage of disease, and a prophylactically effective amount may be more or less than a therapeutically effective amount in some cases.

Medicaments for treating an arthritis may for example include drugs approved by the FDA for treating patients with moderately to severely active rheumatoid arthritis, such as drugs that reduce signs and symptoms of RA and delay structural damage of RA in patients. Such drugs may for example include: nonsteroidal anti-inflammatory drugs (NSAIDs), COX-2 inhibitors such as celecoxib (*Celebrex*) or rofecoxib (*Vioxx*), salicylates, glucocorticoids, TNF inhibitors such as infliximab (*Remicade*), DMARDs such as leflunomide (*Arava*), cyclosporine, mycophenolate mofetil (*Cellcept*), anti-TNF antibodies (as described in US Patnent No. 5,698,195), methotrexate, or soluble versions of the TNF receptor (such as ENBREL(TM), available from Immunex Corp. of Seattle, Washington, USA) or the IL-1 receptor.

In one aspect, the invention relates to the use in gene therapy of an IFN gamma nucleic acid. The IFN gamma nucleic acid may be delivered by a therapeutically acceptable gene therapy vector to modify a patient's IFN gamma allele profile. Gene therapy may for example be used to replace a high risk IFN gamma allele with a low risk IFN gamma allele.

Gene therapy vectors may for example be an adeno-associated vector (AAV). Such a vector may comprise for example: a 5' inverted terminal repeat (ITR); a promoter, such as a CMV enhancer-promoter with a muscle specific enhancer; an intron; a 3'-untranslated region (3'-UTR); a polyadenylation signal, such as an SV40 polyadenylation signal; and a 3'-ITR. For gene therapy vectors, the dosage to be administered may depend to a large extent on the condition and size of the subject being treated as well as the therapeutic formulation, frequency of treatment and the route of administration. Regimens for continuing therapy, including dose, formulation, and frequency may be guided by the initial response and clinical judgment. The parenteral route of injection into the interstitial space of tissue may be preferred, although other parenteral routes, such as inhalation of an aerosol formulation, may

be required in specific administration. In some protocols, a formulation comprising the gene and gene delivery system in an aqueous carrier is injected into tissue in appropriate amounts. The tissue target may be specific, for example the muscle or liver tissue, or it may be a combination of several tissues, for example the muscle and liver tissues. Exemplary tissue targets may include liver, skeletal muscle, heart muscle, adipose deposits, kidney, lung, vascular endothelium, epithelial and/or hematopoietic cells. A nucleic acid of the invention may be delivered to cells *in vivo* using methods such as direct injection of DNA, receptor-mediated DNA uptake, viral-mediated transfection or non-viral transfection and lipid based transfection, all of which may involve the use of gene therapy vectors. Direct injection has been used to introduce naked DNA into cells *in vivo* (see e.g., Acsadi et al. (1991) Nature 332:815-818; Wolff et al. (1990) Science 247:1465-1468). A delivery apparatus (e.g., a "gene gun") for injecting DNA into cells in vivo may be used. Such an apparatus may be commercially available (e.g., from BioRad). Naked DNA may also be introduced into cells by complexing the DNA to a cation, such as polylysine, which is coupled to a ligand for a cell-surface receptor (see for example Wu, G. and Wu, C. H. (1988) J. Biol. Chem. 263:14621; Wilson el al. (1992) J. Biol. Chem. 267:963-967; and U.S. Pat. No. 5,166,320). Binding of the DNA-ligand complex to the receptor may facilitate uptake of the DNA by receptor-mediated endocytosis. A DNA-ligand complex linked to adenovirus capsids which disrupt endosomes, thereby releasing material into the cytoplasm, may be used to avoid degradation of the complex by intracellular lysosomes (see for example Curiel el al. (1991) Proc. Natl. Acad. Sci. USA 88:8850; Cristiano et al. (1993) Proc. Natl. Acad. Sci. USA 90:2122-2126). Defective retroviruses are well characterized for use as gene therapy vectors (for a review see Miller, A. D. (1990) Blood 76:271). Protocols for producing recombinant retroviruses and for infecting cells in vitro or in vivo with such viruses can be found in Current Protocols in Molecular Biology, Ausubel, F. M. et al. (eds.) Greene Publishing Associates, (1989), Sections 9.10-9.14 and other standard laboratory manuals. Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are well known to those skilled in the art. Examples of suitable packaging virus lines include .pψi.Crip, .pψi.Cre, .pψi.2 and .pψi.Am. Retroviruses have been used to introduce a variety of genes into many different cell types, including epithelial cells, endothelial cells, lymphocytes, myoblasts, hepatocytes, bone marrow cells, in vitro and/or in vivo (see for example Eglitis, et al. (1985) Science 230:1395-1398; Danos and Mulligan (1988) Proc. Natl. Acad. Sci. USA 85:6460-6464; Wilson et al.

(1988) Proc. Natl. Acad. Sci. USA 85:3014-3018; Armentano et al. (1990) Proc. Natl. Acad. Sci. USA 87:6141-6145; Huber et al. (1991) Proc. Natl. Acad. Sci. USA 88:8039-8043; Ferry et al. (1991) Proc. Natl. Acad. Sci. USA 88:8377-8381; Chowdhury et al. (1991) Science 254:1802-1805; van Beusechem et al. (1992) Proc. Natl. Acad. Sci. USA 89:7640-7644; Kay et al. (1992) Human Gene Therapy 3:641-647; Dai et al. (1992) Proc. Natl. Acad. Sci. USA 89:10892-10895; Hwu et al. (1993) J. Immunol. 150:4104-4115; U.S. Pat. No. 4,868,116; U.S. Pat. No. 4,980,286; PCT Application WO 89/07136; PCT Application WO 89/02468; PCT Application WO 89/05345; and PCT Application WO 92/07573).

For use as a gene therapy vector, the genome of an adenovirus may be manipulated so that it includes an IFN gamma nucleic acid, but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. See for example Berkner et al. (1988) BioTechniques 6:616; Rosenfeld et al. (1991) Science 252:431-434; and Rosenfeld et al. (1992) Cell 68:143-155. Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (e.g., Ad2, Ad3, Ad7 etc.) are well known to those skilled in the art. Recombinant adenoviruses are advantageous in that they do not require dividing cells to be effective gene delivery vehicles and can be used to infect a wide variety of cell types, including airway epithelium (Rosenfeld et al. (1992) cited supra), endothelial cells (Lemarchand et al. (1992) Proc. Natl. Acad. Sci. USA 89:6482-6486), hepatocytes (Herz and Gerard (1993) Proc. Natl. Acad. Sci. USA 90:2812-2816) and muscle cells (Quantin el al. (1992) Proc. Natl. Acad. Sci. USA 89:2581-2584).

Adeno-associated virus (AAV) may be used as a gene therapy vector for delivery of DNA for gene therapy purposes. AAV is a naturally occurring defective virus that requires another virus, such as an adenovirus or a herpes virus, as a helper virus for efficient replication and a productive life cycle (Muzyczka et al. Curr. Topics in Micro. and Immunol. (1992) 158:97-129). AAV may be used to integrate DNA into non-dividing cells (see for example Flotte et al. (1992) Am. J. Respir. Cell. Mol. Biol. 7:349-356; Samulski et al. (1989) J. Virol. 63:3822-3828; and McLaughlin et al. (1989) J. Virol. 62:1963-1973). An AAV vector such as that described in Tratschin et al. (1985) Mol. Cell. Biol. 5:3251-3260 may be used to introduce DNA into cells (see for example Hermonat et al. (1984) Proc. Natl. Acad. Sci. USA 81:6466-6470; Tratschin et al. (1985) Mol. Cell. Biol. 4:2072-2081; Wondisford et

al. (1988) Mol. Endocrinol. 2:32-39; Tratschin et al. (1984) J. Virol. 51:611-619; and Flotte et al. (1993) J. Biol. Chem. 268:3781-3790). Lentiviral gene therapy vectors may also be adapted for use in the invention.

General methods for gene therapy are known in the art. See for example, U.S. Pat. No. 5,399,346 by Anderson *et al.* (incorporated herein by reference). A biocompatible capsule for delivering genetic material is described in PCT Publication WO 95/05452 by Baetge *et al.* Methods of gene transfer into hematopoietic cells have also previously been reported (see Clapp, D. W., et al., Blood 78: 1132-1139 (1991); Anderson, Science 288:627-9 (2000); and , Cavazzana-Calvo *et al.*, Science 288:669-72 (2000), all of which are incorporated herein by reference).

EXAMPLE 1

48 adult Caucasian patients with severe rheumatoid arthritis and 39 patients with mild rheumatoid arthritis were selected sequentially from a hospital patient population. 50 patients that did not present with symptoms of arthritic disease were selected as a control comparator group. Patients with severe rheumatoid arthritis were aged 58$\pm$ 12 years and were predominantly female and had a mean disease duration of 19$\pm$ 12 years. All such patients had clinically severe polyarticular disease with joint deformities and radiological evidence of subchondral erosions. 75% of such patients had extra-articular manifestations of disease other than the sicca syndrome. 87% of patients with severe rheumatoid arthritis were rheumatoid factor positive. All such patients had not responded favourably to therapy with conventional disease-modifying anti-rheumatic drugs (DMRDS), and had been maintained on cyclosporine treatment for a mean period of 26 months.

Patients with mild disease were aged 61$\pm$ 13 years, predominantly female, and had a mean disease duration of 12$\pm$ 7 years. All such patients hac ..ically mild disease, which had been controlled for a mean period of 90 months by antimalarials alone without prior or current use of DMRDS. Only 26% of such patients had joint deformities, and 36% had extra-articular disease manifestations other than sicca syndrome. 34% of such patients were rheumatoid factor positive.

Peripheral blood was obtained from patients and control subjects, and genomic DNA extracted by proteinase K digestion and by salting out. Molecular typing at the IFN gamma (12q24.12) microsatellite polymorphism was performed by PCR followed by use of a DNA sequencer and gene analysis software. Locus or group-specific amplification was performed using 5' and 3' oligonucleotide amplification primers as follows:

5'6 FAMAG ACA TTC ACA ATT GAT TTT ATT CTT AC 3'

5' CCT TCC TGT AGG GTA TTA TTA TAC G3'

The primers were designed with a high annealing temperature to enhance specificity. Both primers were obtained from Perkin-Elmer-ABI-PRISM and the forward primer was fluorescently labelled at the 5' end.

Genomic DNA (100 ng) was amplified using 50 pmoles each of the oligonucleotide primers, 100 uM, each of dNTP, 1.5 mM $MgCl_2$ and 0.8u of TAQ polymerase in a Perkin-Elmer PCR cycler. Cycling conditions included a 5-minute hot start at 95° C. followed by 32 cycles of 95° C for 45 seconds (denaturation) and 62° C for one minute (annealing and extension) with a final extension of 5 minutes at 62° C in the last cycle. The amplified product was run on a 1.5% agarose gel for detection of positive amplification and then on a long-range gel on a 377 DNA sequencer ABI-PRISM) data were collected using 377 collection software and size analysis was performed using Genescan 2.0.2 software and Genescan 2.0.2 software and Genescan-500 ROX as a size standard (ABI-PRISM).

A total of six alleles were documented in the patients and controls, ranging in length from 120 bp to 130 bp, as shown in Table 1.

TABLE 1: Proportion of subjects expressing individual alleles in the first intron of the IFNγ gene. Controls (n=50), patients with severe RA (n=48), patients with mild RA (n=39).

IFNγ Alleles (Size)	Controls	Severe RA			Mild RA				
	%	%	OR[a]	P[a]	%	OR[a]	p[b]	OR[b]	p[b]
A2 (130 bp)	0	6	7.77	NS	0	-	-	6.08	NS
A3 (128 bp)	16	15	0.90	NS	15	0.95	NS	0.94	NS
A4 (126 bp)	12	73	19.74	p<0.0001	21	1.89	NS	10.43	p<0.0001
A5 (124 bp)	68	77	1.58	NS	67	0.94	NS	1.68	NS
A6 (122 bp)	68	6	0.019	p<0.0001	64	0.50	NS	0.037	p<0.0001
A7 (120 bp)	0	0	-	-	3	3.93	NS	0.27	NS

[a] severe or mild RA compared with controls
[b] severe compared with mild RA
NS: not statistically significant
OR: odds ratio

The frequency of the polymorphisms in normal subjects ranged from 0% for the 130 bp and 120 bp alleles, to 68% for the 122 bp allele. The genotype frequencies did not deviate from the expected value by Hardy-Weinberg equilibrium. The alleles identified herein appear to correspond closely in length to those initially reported by Ruiz-Linares, which ranged from 122-134 bp (Ruiz-Linares, 1994 Hum. Mol. Genet. 2(9):1508). Such alleles may vary, for example, depending upon the ethnic origin of the subjects and the methodology of characterization (see for example Awata, T. et al. 1994 Diabetologia 37:1159). In some populations, 6-8 alleles encompassing at least 11-15 CA repeats may exist at this site, including the intermediate polymorphism of 128 bp.

As shown in this example, patients with severe rheumatoid arthritis differ significantly in the frequency of 2 alleles. The 126 bp allele was present in 73% of patients with severe rheumatoid arthritis compared with 21% of patients with mild rheumatoid arthritis (OR: 10.43, $p < 0.0001$) and 12% of normal subjects (OR: 19.74, $p < 0.0001$). In contrast, the 122 bp allele was detected in only 6% of patients with severe rheumatoid arthritis compared with 64% of patients with mild disease (OR: 0.037, $p < 0.0001$) and 68% of normal subjects (OR: 0.019, $p < 0.0001$). There was no significant difference in the

frequencies of the other microsatellite polymorphisms between the three groups of individuals.

Table 2 shows a grouping of the subjects into one of four categories, depending upon their expression of the 126 bp allele and the 122 bp allele. Almost three quarters (73%) of patients with severe rheumatoid arthritis expressed the 126 bp allele without the 122 bp allele, compared with 10% of patients with mild rheumatoid arthritis (OR: 23.56, $p < 0.0001$) and 4% of normal subjects (OR: 60.62, $p < 0.0001$). In contrast, only 6% of patients with severe rheumatoid arthritis expressed the 122 bp allele without the 126 bp allele compared with 54% of patients with mild rheumatoid arthritis (OR: 0.057, $p < 0.0001$) and 70% of normal subjects (OR: 0.029, $p < 0.0001$). The 126 bp and 122 bp alleles were not conjointly expressed by any patients with severe disease compared with conjoint expression in 10% of patients with mild disease (OR: 0.081, p = NS) and conjoint expression in 8% of normal subjects (OR: 0.11, p = NS).

Table 2: Proportion of subjects expressing the 126 bp allele, 122 bp allele, both or neither in the first intron of the IFNγ gene. Controls (n=50), patients with severe RA (n=48) patients with mild RA (n=39).

Categories	Controls	Severe RA			Mild RA				
	%	%	OR[a]	p[a]	%	OR[a]	p[a]	OR[b]	p[b]
126 bp allele	4	73	60.62	<0.0001	10	2.74	NS	23.56	<0.0001
122 bp allele	70	6	0.029	<0.0001	54	0.50	NS	0.057	<0.0001
Both	8	0	0.11	NS	10	1.31	NS	0.081	NS
Neither	18	21	1.20	NS	26	1.57	NS	0.76	NS

a compared with controls
b severe compared with mild
NS: not statistically significant
OR: odds ratio

Table 3: Results of logistic regression for the effects of HLA-DR, IFN-g, and clinical measures on the odds of severe disease. Odds ratios and chi-square statistics are marginal (i.e. have been adjusted for all other factors). Odds ratios here reflect the distribution of patients observed rather than underlying prevalence of mild and severe RA. Controls (n=50), patients with severe RA (n=48) patients with mild RA (n=39).

Factor	**d.f.**	**Severe vs. Control** x^2	**Severe vs. Mild** x^2	**Severe vs. Mild** x^2
HLA-DR	3	11.23*	13.04**	10.66*
IFN-γ	3	65.88**	43.91***	28.09***
RF	2	--	--	9.33**
Age	1	--	--	0.46
Duration	1	--	--	6.57*
Gender	1	--	--	3.84**

Factor	**Effect**	**O.R.**	**O.R.**	**O.R.**
HLA-DR	H vs. L	23.95*	14.55	48.27
	B vs. L	7.23	0.43	0.63
	N vs. L	2.10	2.15	2.52
IFN-γ	H vs. L	327.09***	107.57***	278.02**
	B vs. L	0.03	0.00	0.00
	N vs. L	19.26***	5.13*	9.95
RF	NA vs. neg			2.10
	pos vs. neg			25.07*
Age	10 years			0.77
Duration	10 years			4.71*
Gender	m vs. f			11.16

*p<0.05; **p<0.01 level; ***p<0.001 level.
HLA-DR: H = QKRRA/QRRAA, L = DERAA, B = Both, N = Neither
IFN-g: H = 126 bp allele; L = 122 bp allele; B = Both; N = Neither

These striking results were confirmed in a subsequent and independent group of 12 patients with severe RA who were selected according to the same clinical and laboratory criteria. Seventy-five percent of these patients expressed the 126 bp allele and 8% the 122 bp

allele; when all subjects with severe RA were combined (n=60) the patient frequencies were unchanged from those reported in tables 1 and 2.

Logistic regression was used to examine the influences of IFN-γ polymorphism, HLA DR-B1 genotype (Wayland and Goronzy, 1997, J. Mol. Med. 75:772) and other prognostic factors. The results are shown in table 3. Inheritance of the INF-γ 126 bp allele is strongly associated with the presence of severe RA even after accounting for HLA-DRB1 polymorphism, while possession of the IFNγ 122 bp polymorphism is highly negatively associated with the presence of severe disease. The association of these IFNγ alleles with severe RA is considerably greater than that noted for the most tightly associated MHC class II alleles or other clinical predictors including gender, age at onset, duration of disease, or rheumatoid factor positivity.

In accordance with one aspect of the present invention, the diagnostic test for the presence of IFN gamma alleles may be carried out on asymptomatic individuals to assess the individual's susceptibility to rheumatoid arthritis. In individuals presenting with arthritic symptoms, the test may be utilized to assess the likelihood of progression to the severe form of the disease. In accordance with these aspects of the invention, the presence of the 126 bp allele may be taken as an indication of increased susceptibility to rheumatoid arthritis, including increased susceptibility to progression of the arthritis to the severe form of the disease, as set out in Table 3.

The diagnostic test may in some embodiments be utilized to determine whether the IFN gamma alleles are homozygous or heterozygous for example, in the exemplary embodiment, 10% (6/60) of patients with severe rheumatoid arthritis were homozygous for the 126 bp allele, compared with 3% (1/39) of those with mild disease and 0% (0/50) of normal controls. In contrast, none of the 60 patients with severe rheumatoid arthritis were homozygous for the 122 bp allele, compared with 8% (3/39) of those with mild disease and 14% (7/50) of normal controls.

EXAMPLE 2

As shown in Figures 1, 2 and 3, 87 patients were segregated into three groups on the basis of their *HLA-DRB1* alleles, on the basis of the charge on the amino acid at position 71, being positive ("+", i.e. lysine (K), arginine (R) or histidine (H)), negative ("-", i.e. glutamate (E) or aspartate (D)) or neutral ("0", the remaining amino acids). Figure 2 shows the HLA-DRB1 amino acid sequences 70-74 for both alleles for patients with severe RA. Figure 3 shows the HLA-DRB1 amino acid sequences 70-74 for both alleles for patients with mild RA. Individuals are segregated in the chart of Figure 1 into three groups based on their HLA-DRB1 sequence:

i) A+/+ individuals are homozygous for a positive amino acid at position 71;

ii) A+/0 individuals are heterozygous, having a positive amino acid allele and a neutral amino acid allele;

iii) A-/* individuals have at least one copy of an allele with a negative amino acid at position 71.

With respect to the IFN-gamma alleles in the chart of Figure 1 "+" indicates a high risk allele, while "-" indicates a low risk allele. For example, individuals who are homozygous for the high risk allele are grouped on the branch of the tree denoted by "+/+".

The risk analysis chart indicates, for example, that patients homozygous a positive amino acid at position 71 of HLA-DRB1 and homozygous for the high risk IFN allele suffer from severe RA in 24 out of 26 cases, while patients having at least one HLA-DRB1 allele with a negative amino acid (shown as "-/*) who are also homozygous for the low risk IFN allele ("-/-) present with mild RA in 7 out of 7 cases.

Although various embodiments of the invention are disclosed herein, many adaptations and modifications may be made within the scope of the invention in accordance with the common general knowledge of those skilled in this art. Such modifications include the substitution of known equivalents for any aspect of the invention in order to achieve the same result in substantially the same way. Numeric ranges are inclusive of the numbers defining the range. In the claims, the word "comprising" is used as an open-ended term, substantially equivalent to the phrase "including, but not limited to".